VIROLOGY 216, 35–45 (1996)
ARTICLE NO. 0032

Epithelial Cells Display Separate Receptors for Papillomavirus VLPs

YING MEI QI, SHI WEN PENG, KYLIE HENGST, MAGNUS EVANDER, DAVID S. PARK,[1]
JIAN ZHOU,[2] and IAN H. FRAZER[3]

Papillomavirus Research Unit, University of Queensland, Princess Alexandra Hospital, Woolloongabba, Queensland 4102, Australia

Received July 25, 1995; accepted November 29, 1995

We examined the distribution of putative receptors for papillomavirus (PV) capsid proteins on various cell types, using either Hexahis HPV6b L1 fusion protein or synthetic HPV6b virus-like particles (VLPs). Specific, saturable binding of VLPs to CV-1 cells was demonstrated using ^{35}S-labeled VLPs, with an average receptor number of 1×10^4/cell and a binding affinity constant (K_a) of 4×10^7 *M*. VLP binding was quantitated by flow cytometry using a monoclonal antibody to the L1 capsid protein. Intense staining of epithelial and mesenchymal cells was observed. Some immature bone marrow-derived cells bound VLPs weakly, while the majority of B lymphoma cells demonstrated no binding. Binding to 12 of 16 VLP receptor positive cell lines was abolished by trypsin pretreatment of cells. Removal of cellular sialic acid or O-linked oligosaccharides separately did not affect VLP binding, which was enhanced about 25% when cells were pretreated with both neuraminidase and *O*-glycosidase. Culture of cells with sufficient tunicamycin to inhibit Concanavalin A binding did not diminish the binding of VLPs. Denatured L1 protein, either from VLPs or expressed from *Escherichia coli* as a Hexahis fusion protein, bound to a trypsin-resistant structure on a range of cell types and did not block the binding of VLPs to cells. Dual-fluorescence assay with a Burkitt lymphoma line BL72 demonstrated that Hexahis L1 protein and VLPs bind to separate cell surface molecules on BL72 cells. We conclude that the first binding of PV virus to cells is via a widely distributed membrane protein receptor(s) and that subsequent processing of particles may involve other non-trypsin-sensitive structure(s) also displayed on the cell membrane.

INTRODUCTION

The initial event in viral infection involves the attachment of virus to the host cell. Some viruses, such as herpes simplex virus (Bouayyad and Menezes, 1990), have a broad host cell range. Other viruses, such as human immunodeficiency virus (Wong-Staal and Gallo, 1985) and measles virus (Maisner *et al.,* 1994), have a narrow host cell range due to restricted virus attachment. Most viruses appear to attach to cell membranes via cell surface proteins. There are some well-characterized examples, including the CD4 molecule for human immunodeficiency virus type 1 (Dalgleish *et al.,* 1984) and the C3d receptor CR2 for Epstein–Barr virus (Fingeroth *et al.,* 1984).

Papillomaviruses (PVs) present a clinical problem because of their association with anogenital cancer. PVs selectively infect epithelial cells, while some are also able to transform fibroblasts, but difficulties in propagating PV *in vitro* have hindered study of the pathway of viral uptake and the interactions between the virus capsid and putative cell membrane receptors.

Authentic PV capsids include two virus-encoded capsid proteins, L1 and L2, and contain some cellular histone associated with the ≈7.9-kb double-stranded DNA viral genome. L1 protein when expressed in eukaryotic expression systems is able to self-assemble into virus-like particles (VLPs) *in vitro* (Zhou *et al.,* 1991; Kirnbauer *et al.,* 1992; Park *et al.,* 1993). L1 protein expressed in prokaryotic expression systems does not, in contrast, have the capacity to assemble into VLPs. Recently, PV VLPs have been used to characterize the binding of PVs to cells: using a range of assay techniques, binding has been demonstrated to be saturable and to involve a trypsin-sensitive receptor structure on the cell membrane (Roden *et al.,* 1994; Muller *et al.,* 1995; Volpers *et al.,* 1995); to date, no cell has been reported to be unable to bind VLPs. Some viruses bind to more than one molecule on the host cell surface (Wickham *et al.,* 1993; Stevenson *et al.,* 1995), and these may have different functions in virus processing. To determine whether there were cells without VLP receptors, and whether PV binding to cells might involve more than one receptor structure, we compared the binding of PV VLPs and soluble PV L1 protein to the cell membrane of a range of cell types using HPV6b VLPs expressed in insect cells, and Hexahis L1 protein expressed in *Escherichia coli.*

[1] Current address: UCSD, School of Medicine-0677, 9506 Gilman Drive, La Jolla, CA 92093-0677.

[2] Current address: Research Division, Dept. of Ob/Gyn, Loyola University Medical Centre, Maywood, IL 60156.

[3] To whom correspondence and reprint requests should be addressed. Fax: 61-7-240-2048. E-mail: ifrazer@gpo.pa.uq.oz.au.

0042-6822/96 $12.00

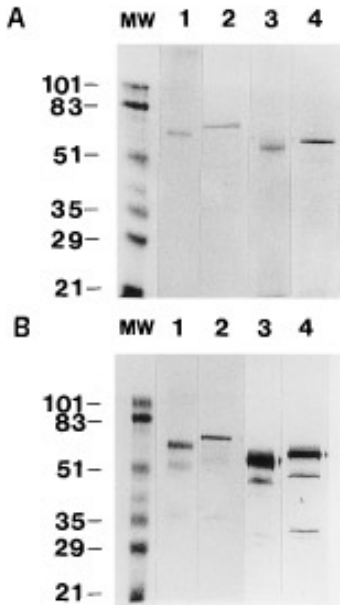

FIG. 1. (A) SDS–PAGE analysis of purified HPV6bL1 VLP and HPV6bL1 Hexahis fusion protein. MW, marker lane; lane 1, reduced HPV6bL1 Hexahis fusion protein; lane 2, unreduced HPV6bL1 Hexahis fusion protein; lane 3, reduced HPV6bL1 VLPs; lane 4, unreduced HPV6bL1 VLPs. (B) Immunoblot of the proteins from panel A. Proteins were detected with rabbit polyvalent antiserum raised against HPV6bL1 Hexahis fusion protein and demonstrated with peroxidase conjugated sheep anti-rabbit antiserum.

MATERIAL AND METHODS

Preparation of HPV6b L1 capsid protein and VLPs

The L1 open reading frame (ORF) of HPV6b was excised from pHPV6b and cloned into the *Bam*HI/*Hin*dIII sites of the N-terminal Hexahis fusion protein expression vector pTrcHis (Invitrogen) to produce pTrcHis6bL1. Expression of HPV6bL1 hexahis fusion protein was induced in log-phase culture of pTrcHis6bL1 transformed *E. coli* with 1 m*M* IPTG for 4–6 hr. To purify the Hexahis L1 protein, bacteria, washed in phosphate-buffered 0.15 *M* saline, pH7.4 (PBS), were suspended in guanidinium lysis buffer and sonicated. Hexahis L1 protein was partially purified from the clarified bacterial sonicate by nickel chelate affinity chromatography and eluted from the nickel resin using a continuous pH gradient (pH 7.8 to 4.0). Fractions containing Hexahis L1 were pooled, precipitated with acetone, and dried under nitrogen. This pooled protein was further purified by preparative SDS–PAGE (Bio-Rad Prep Cell) using a 10% separating gel. Eluate fractions with a single Hexahis L1 protein band were dialyzed against PBS, and L1 protein was precipitated with acetone and dried under nitrogen. The dried protein was dissolved in sterile PBS for further use (Fig. 1, lanes 1 and 2).

Construction of HPV6b L1 recombinant baculovirus (BV) was carried out essentially as described for HPV16L1 (Park *et al.,* 1993). The L1 ORF of HPV6b was excised from pHPV6b and cloned into the *Bam*HI/*Sma*I sites of pVL1393 to produce the plasmid pVL6bL1. This intermediate vector, together with viral DNA from AcNPV, was used to create recombinant BV vAc6L1 which were plaque purified and characterized for L1 expression. VLPs were prepared (Kirnbauer *et al.,* 1992) and purified (Zhou *et al.,* 1991) from vAc6L1-infected Sf9 cells essentially as described. Sf9 cells were infected with vAc6L1 recombinant baculovirus at m.o.i. of 2. At 96 hr postinfection cells were harvested, resuspended in 10 ml PBS, and homogenized. A 100 *g* pellet was resuspended in PBS with protease inhibitors and sonicated. Sonicates were subjected to centrifugation at 10,000 *g* for 30 min, resuspended, and subjected to discontinuous density gradient centrifugation through 40% (wt/vol) sucrose. The resuspended pellet underwent continuous density gradient centrifugation in 1.3 g/ml CsCl. A particulate band was harvested, dialyzed against PBS, and analyzed by SDS–PAGE and immunoblot (Fig. 1, lanes 3 and 4). Purified VLPs were adsorbed to carbon-coated grids, stained with uranyl acetate, and examined by Hitachi H-800 electron microscopy.

Irreversible denaturation of VLPs

Purified VLPs were denatured by a modification of method of Schindler *et al.* (1995). VLPs were dialyzed against 0.1 *M* Tris/HCl buffer, 10 m*M* EDTA, pH 8.3, at 4° overnight. Powered guanidine hydrochloride was added to a final concentration of 6 *M.* Ten microliters of 100 m*M* dithiothreitol was added and the mixture held at 50° for 1.5 hr. Freshly prepared 0.5 *M* iodoacetamide was added to a final concentration of 10% (vol/vol), and the mixture held at room temperature in the dark for a further 1.5 hr. The mixture was then dialyzed extensively against PBS.

Antibodies

HPV16 L1-specific monoclonal antibody (MAb) Camvir 1 (McLean *et al.,* 1990) was obtained from Dr. Margaret Stanley, University of Cambridge. A polyclonal murine antiserum was raised to HPV6bL1 VLPs by immunizing a C57Bl/6 mouse three times subcutaneously at 2-week intervals with 100 μg of purified baculovirus-expressed HPV 6bL1 VLPs in PBS. Rabbit antiserum to HPV6bL1 Hexahis fusion protein was prepared by immunizing a rabbit three times subcutaneously at 2- to 4-week intervals with 100 μg of purified HPV6bL1 Hexahis fusion protein with Freund's adjuvant (Muller *et al.,* 1995). Rabbit antiserum to HPV6bL1 VLPs was raised by immunizing a rabbit three times subcutaneously at 2-week intervals with 100 μg of purified baculovirus-expressed HPV6bL1 VLPs in PBS.

Cell lines

Cell lines (Table 1) were obtained from ATCC, from Dr. Isiah Fidler (KM12C) or from Dr. Anne Kelso, Dr. Ihor

TABLE 1

Binding of HPV6b VLPs to Various Cell Lines

Cell	Lineage	Species	Fc receptor status	VLP binding[a]	VLP binding after trypsin
STO	Fibroblast	Human	−	++	−
K562	Myeloid	Human	−	++	−
FD EP-1	Hemopoietic	Mouse	−	−	−
WEHI-279.1	B lymphoma	Mouse	+	−	−
7Td-1	Hybridoma	Mouse	+	++	−
CTLL	T lymphocyte	Mouse	−	+	−
32D	Immature mast	Mouse	−	+	++
EL4	Thymoma	Mouse	−	++	++
CI80135	Epithelial–ovarian	Human	+	++	++
KM12C	Epithelial–colon	Human	−	++	−
Lisp1	Epithelial–colon	Human	−	+	−
SW480	Epithelial–colon	Human	−	++	−
CV1	Epithelial	Monkey	−	+++	−
DG75	B lymphoma	Human	−	−	−
BL72	B lymphoma	Human	−	++	++
MM962	Melanoma	Human	−	++	−
SHSY5Y	Neuroblastoma	Human	−	++	−
HUVEC	Endothelial	human	−	+	−
SF9	Coelomic	Insect	−	++	−

[a] Scored as −, no cell positive; +, a few cells positive (<10%); ++, substantial binding (10–50%); +++, most cells positive (50–100%).

Misko, Dr. Mike Watters, or Dr. Georgia Trench from Queensland Institute of Medical Research, Australia.

Binding assay

Binding of VLPs to cells was measured by a modification of the procedure of Borrow and Oldstone (1992). Cells were washed and suspended at 10^6/ml in 2% bovine serum albumin (BSA; Sigma, NJ) in PBS. Adherent cells were detached by brief exposure to 2.5 g/liter trypsin, 0.2 g/liter EDTA, washed once in DMEM/10% FBS, and incubated in suspension with gentle agitation every 15 min for 2 hr at 37° in water-saturated 95% air/5% CO_2 prior to analysis. All subsequent procedures were carried out on ice. HPV6bL1 Hexahis fusion protein or HPV 6bL1 VLPs at various concentrations were added to aliquots of 2×10^5 cells. The mixture was held for 60 min. Cells were then washed three times in PBS/2% BSA and held for 30 min with 200 μl of MAb (1:20) or polyvalent antiserum (1:100 to 1:250). Cells were washed three times in PBS with 2% BSA and held with sheep anti-mouse or anti-rabbit IgG (FITC conjugated, Sigma) for a further 30 min. After three further washes cells were resuspended in 200 μl of "FACS fix" buffer (40% formaldehyde, 2.5% glucose, 0.2% sodium azide). Appropriate controls in each experiment and with each cell line omitted each antibody or utilized a suspension of insoluble baculovirus recombinant HPV16 E7 protein and a MAb (8F) specific for HPV16 E7 (Tindle *et al.,* 1990) in lieu of VLPs and anti-L1 antibodies, or a recombinant Hexahis fusion protein (merozoite surface antigen protein MSA-1, a gift of Dr. John Cox, CSL, Melbourne) in lieu of Hexahis L1.

Trypsin treatment of cells in suspension was carried out prior to assay of binding, as described by Horton and Burand (1993). Trypsin was added at 1 mg/ml in total 0.2 ml, and cells were held for 15 min at 22°. Cells were washed in 10% FBS in PBS and resuspended in 2% BSA in PBS.

Dual-fluorescence stain binding assay

BL72 cells (2×10^5) in 200 μl PBS/2% BSA were exposed to 25 μg VLPs for 60 min. Cells were washed with ice-cold PBS/2% BSA between each subsequent addition of antiserum or antigen. Rabbit anti-HPV6bL1 antiserum (1:100) was added for 30 min, followed by anti-rabbit Ig–TRITC (Sigma). The cells were then further reacted with a complex of Hexahis 6bL1 plus anti-Tag MAb (Invitrogen) for 30 min. Finally, cells were exposed to anti-mouse Ig–FITC (Sigma) for 30 min. Cells were resuspended in 200 μl of FACS fix buffer. Controls omitted each of the antigens or antisera in turn.

Cell labeling and competition, saturation studies

VLPs were labeled with [^{35}S]methionine by modification of a described procedure (Sahli *et al.,* 1993). Briefly, Sf9 cells were infected by recombinant baculovirus Ac6bL1 at a multiplicity of infection of 10. At 18 hr postinfection, the cells were resuspended in Grace's insect cell culture medium without methionine supplemented with 10% dialyzed FBS. [^{35}S]Methionine (0.5 mCi) (ICN Biochemical Inc.) was added to 2×10^8 cells. After 9 hr unlabeled methionine was added, and cells were cultured for another 36 hr. Labeled VLPs were purified as

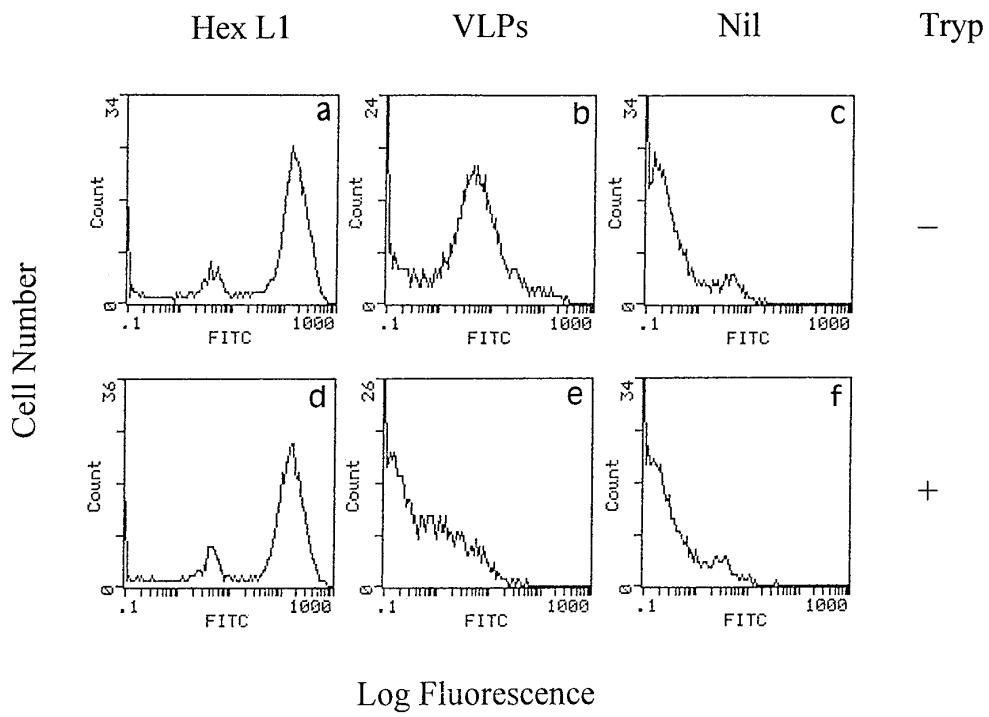

FIG. 2. Binding of HPV6bL1 VLPs, and of HPV6bL1 Hexahis fusion protein, to CV-1 cells. Cells in suspension were exposed to Hexahis L1 (a, d) or VLPs (b, e) or were exposed to antibodies only (c, f), and VLP binding was determined with Camvir 1 MAb and FITC-labeled anti-mouse Ig antiserum. Cells were either exposed to trypsin before assay (d, e, f) or not so exposed (a, b, c).

described above. For competition binding studies 160 ng of [^{35}S]methionine-labeled VLPs (specific affinity, 36 nCi/μg) were added together with a variable quantity of unlabeled 6bL1 VLPs or unlabeled 6bL1 Hexahis fusion protein to 2×10^5 CV-1 cells for 1 hr on ice. For saturation studies, a variable quantity of labeled VLPs was added to 2×10^5 CV-1 cells for 2 hr on ice. The radioactivity present in the supernatant and bound to the cells was determined by liquid scintillation (MINAXIβ Tri-Carb$_{(R)}$ 4000 series).

Enzyme treatment of membrane proteins

CV-1 cells were suspended at 2×10^5/200 μl of PBS/2% BSA. Control aliquots were subjected to the same procedures without the addition of enzyme.

Neuraminidase. Four microliters of 50 U/ml neuraminidase (Sigma, St. Louis) was added to cells and held at 37° for 1 hr (Borrow and Oldstone, 1992; Keppler *et al.,* 1994).

O-glycosidase. Ten microliters of 0.5 U/ml *O*-glycosidase (Boheringer) was added to cells and held at 37° for 1 hr (Borrow and Oldstone, 1992; Maisner *et al.,* 1994).

Neuraminidase + O-glycosidase. Cells treated with neuraminidase as above were washed twice with PBS and resuspended in 200 μl of PBS containing 10 μl of 0.5 U/ml *O*-glycosidase. Cells were then held at 37° for 1 hr (Borrow and Oldstone, 1992; Maisner *et al.,* 1994).

Treated and control cells were washed in PBS/2% FBS three times and resuspended in PBS/2% FBS containing 25 μg VLPs. Binding of VLPs was analyzed as above.

Tunicamycin treatment of cells

Tunicamycin (Sigma) was dissolved in 0.01 *N* NaOH at 5 mg/ml and diluted in ddH_2O to a stock concentration of 50 μg/ml (Heifetz *et al.,* 1979). CV-1 cells in the logarithmic growth phase were diluted in 1:10 in fresh DMEM + 10% FCS without or with various concentrations of tunicamycin (Keppler *et al.,* 1994). After 48 hr of culture with inhibitor, cells were counted and cell viability was determined by trypan blue exclusion. Cells (2×10^5) were washed in PBS/2% BSA and analyzed further for binding of HPV 6bL1 VLPs, 6bL1 Hexahis fusion protein, or Concanavalin A.

RESULTS

Binding of VLPs and L1 protein to epithelial cells

Adherent CV-1 epithelial cell monolayers were exposed to HPV6b L1 VLPs (VLPs), HPV6b L1 Hexahis fusion protein (Hexahis L1), or an irrelevant BV-derived papillomavirus protein (HPV16 E7). Binding of particles was sought by indirect immunofluorescence with an HPV16L1-specific MAb (Camvir 1) which cross-reacts with HPV6bL1, and FITC-conjugated anti-mouse Ig. Fine particulate membrane staining was observed on cells held at 4°, and this staining coalesced after incubation

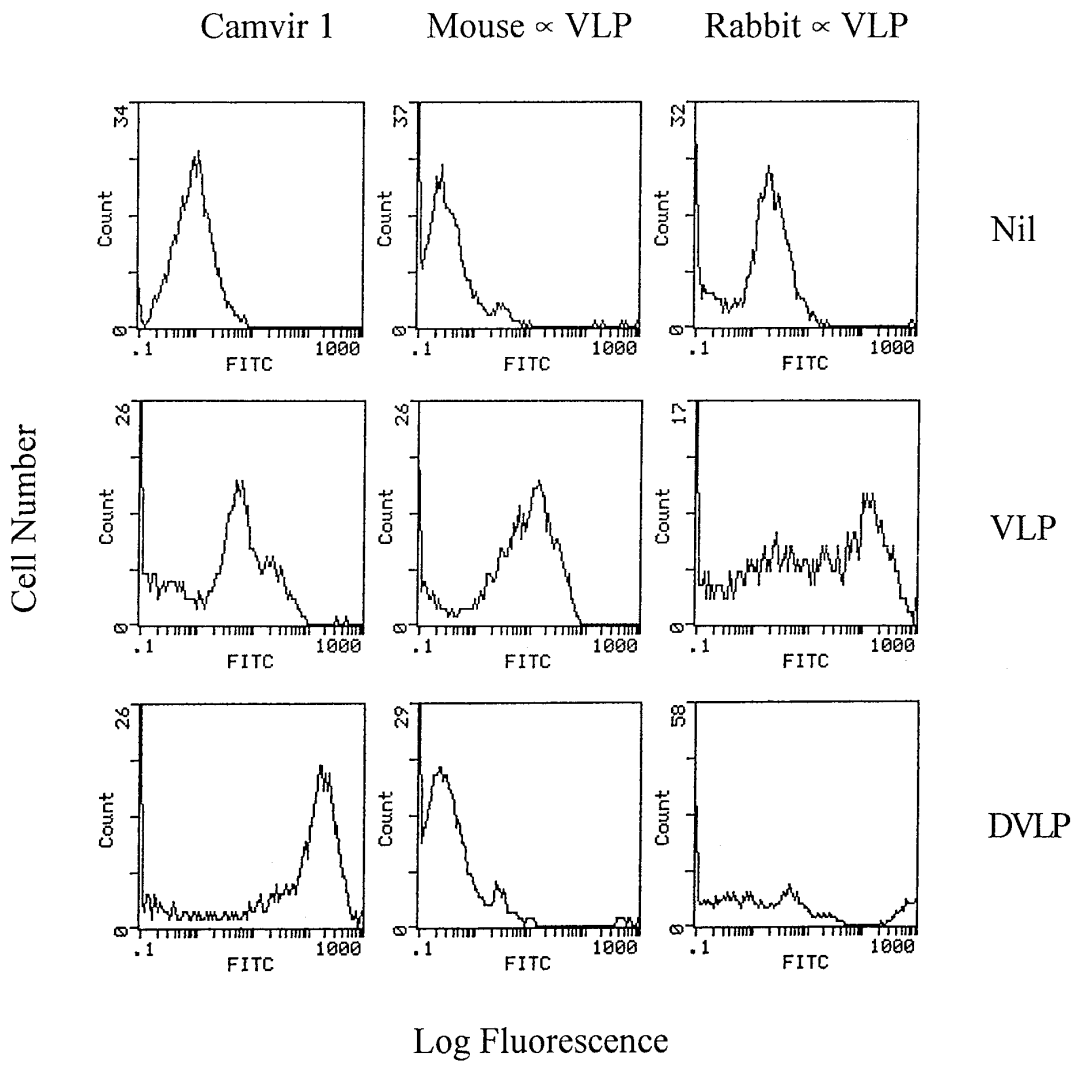

FIG. 3. Flow cytometric analysis of binding of native and denatured HPV6bL1 VLP protein to CV-1 cells, showing that both preparations bind and binding of each can be distinguished by appropriate antisera. CV-1 cells in suspension were exposed to antibodies only (Nil), to BV recombinant HPV6bL1 VLPs (VLP), or to denatured L1 protein derived from HPV6bL1 VLPs (DVLP). Binding of L1 protein was examined in each case with three primary antibodies: a monoclonal antibody specific for a linear determinant shared between HPV6b and HPV16 (Camvir 1) which recognizes both native and denatured L1, a polyvalent murine antiserum raised against VLPs without adjuvant (Mouse ∝ VLP) which recognizes native but not denatured L1, or a rabbit antiserum raised against VLPs without adjuvant (rabbit ∝ VLP) which recognizes both native and denatured L1.

of the cells at 37° for 1 hr into larger punctate aggregates, suggesting receptor cross-linking. CV-1 cells in suspension were examined for Hexahis L1 or VLP binding with Camvir 1 by flow cytometry and were demonstrated to bind both Hexahis L1 and VLPs (Fig. 2). To confirm that this assay could measure binding of VLPs as opposed to denatured L1, these experiments were repeated with an antiserum raised against VLPs without adjuvant, and previously demonstrated specific by ELISA for VLPs but not denatured L1 protein (data not shown). This antiserum bound to CV-1 cells exposed to VLPs but not to cells exposed to denatured VLP-derived L1 protein (Fig. 3). DG75 cells were unable to bind VLPs, but some were able to bind L1 protein from denatured VLPs (Fig. 4), confirming that the assay could distinguish between binding of Hexahis L1 and binding of VLPs, and demonstrating that some cultured cell lines are unable to bind PV VLPs.

Specificity of binding of labeled and unlabeled VLPs

To confirm that HPV6bL1 VLP bound to cells by a specific and saturable mechanism, unlabeled VLP and Hexahis L1 were tested for their ability to compete with the binding of ^{35}S-labeled VLPs to CV-1 cells. Cells on ice were exposed to various amounts of unlabeled VLP mixed with 160 ng of ^{35}S-labeled VLPs, and the amount of cell bound [^{35}S] was determined (Fig. 5a). Unlabeled VLPs effectively competed the binding of labeled VLPs to CV-1 cells. Binding was reduced to 20% of maximal, by an 81-fold excess of unlabeled VLPs. In contrast, a similar (mass/mass) excess of unlabeled Hexahis L1,

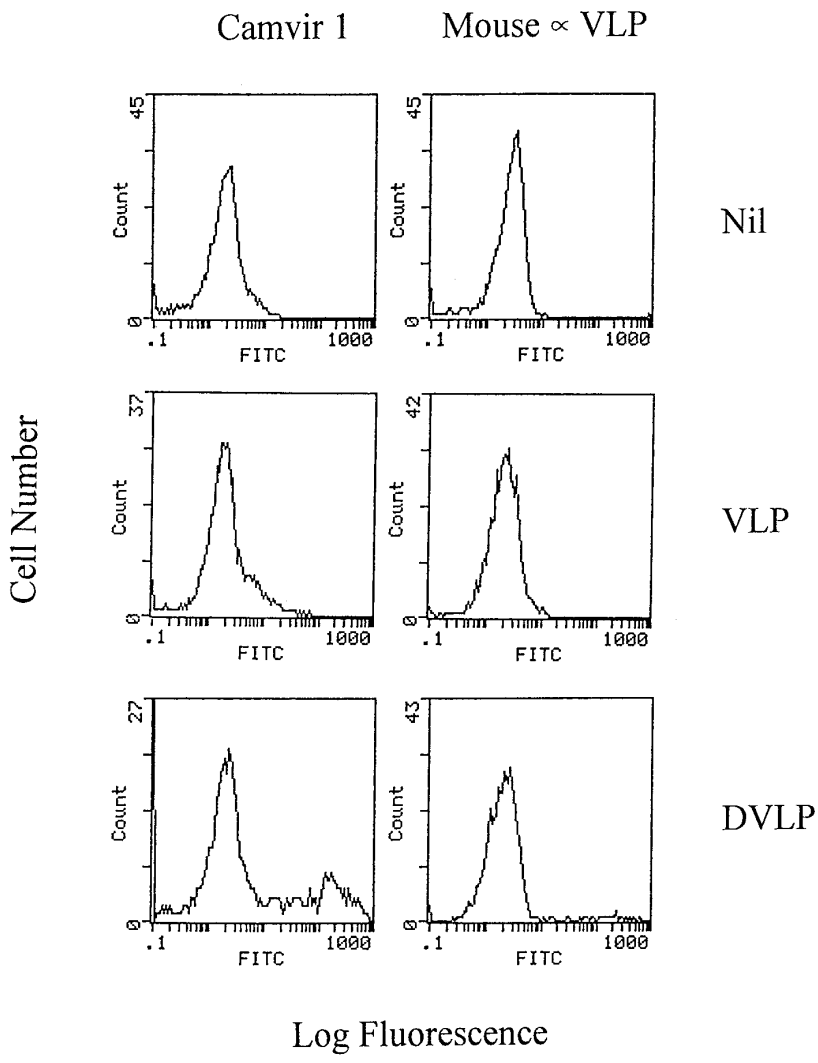

FIG. 4. Flow cytometric analysis of binding of VLPs and of denatured L1 from VLPs to DG75 cells which are VLP receptor negative. The same methods, VLP preparations, and antibodies were used as in Fig. 3.

representing a ~3000-fold molar excess of Hexahis L1 over VLPs, had no effect on the binding of labeled VLPs.

Distribution of the PV receptor on various cell lines

A range of cell lines derived from epithelial, mesenchymal, neural, and hemopoetic lineages were tested for binding of VLPs. The most intense binding per cell was seen with CV-1 cells (Table 1). By flow cytometric analysis, a wide range of cells showed significant binding of the VLPs when exposed to 20 μg VLP per 2×10^5 cells, but a hemopoetic cell and some B lymphoma cells showed no specific binding even at higher concentrations of HPV VLPs.

Number of VLP receptor sites per CV-1 cell

Using apparently saturating concentrations of labeled VLPs, the number of ^{35}S-labeled VLPs bound per CV-1 cell was calculated. As 2×10^5 cells were able to bind 160 ng of labeled VLPs, at least 7.4×10^3 receptor sites were present per CV-1 cell. To confirm the receptor number and affinity on CV-1 cells, Scatchard analysis was performed using graduated amounts of labeled VLPs, and also using graduated amounts of unlabeled VLPs to compete the binding of a fixed amount of labeled VLPs (Fig. 5b). These analyses predicted a VLP receptor density of 1.03×10^4/cell, and a molar binding affinity (K_a) for L1 of 4×10^7 *M,* or for VLPs of 1.4×10^{10} *M.*

Specificity of binding for particulate L1 and VLPs

The BL72 cell line, which exists in two states of differentiation, was observed to bind Hexahis L1 strongly and VLPs weakly. Dual-color fluorescence analysis (Fig. 6) indicated that BL72 cells could be separated into a population binding Hexahis L1 and a population binding VLPs: relatively few cells (7%) bound both. When Hexahis L1 and VLP were present together, the relative number of

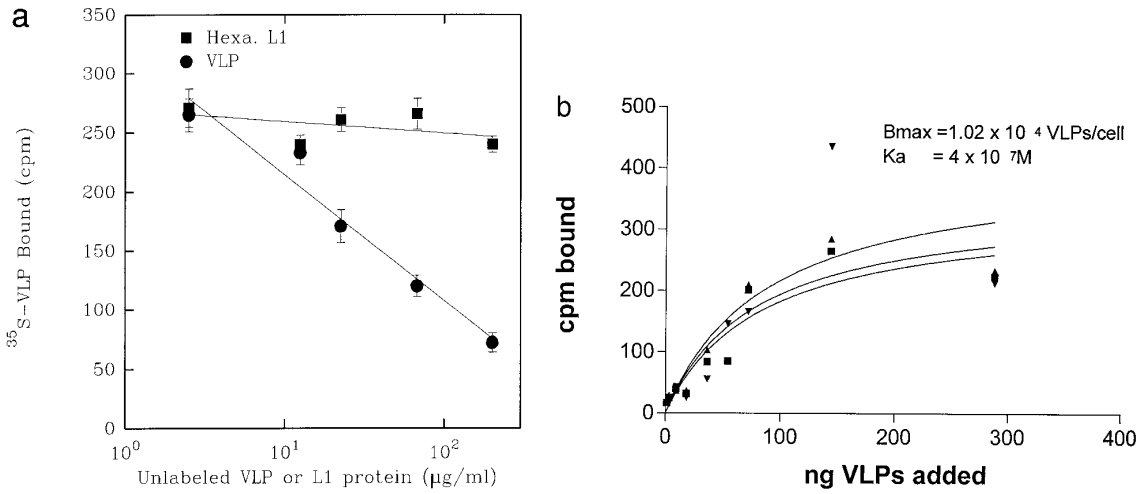

FIG. 5. Binding of VLPs to CV-1 cells. (a) 160 ng of ^{35}S-labeled VLPs (1262 cpm) were mixed with a series of dilutions of unlabeled VLPs (●, mean ± 1 SEM of three experiments) or of soluble hexahis L1 (■, mean ± 1 SEM of two experiments). 2×10^5 CV-1 cells were exposed to the VLPs for 60 min on ice, washed, and bound [^{35}S] quantitated. (b) CV-1 cells were exposed to increasing concentrations of ^{35}S-labeled VLPs and binding quantitated as for (a). B_{max} and K_m were calculated assuming "single receptor" binding kinetics. ▲, ▼, and ■ represent three independent experiments.

cells binding each ligand (66% for Hexahis L1 and 10% for VLP) was similar to that observed with each ligand alone, indicating that binding of Hexahis L1 or VLP was not inhibited by the addition of VLP or Hexahis L1. Taken together, these data support the hypothesis that Hexahis L1 and synthetic VLPs react with different receptors on the cell membrane of epithelial cells and suggest that expression of these receptors may depend on the state of differentiation of the cell, as BL72 is known to be found in culture in both a differentiated and a relatively undifferentiated state.

Binding of VLPs and Hexahis L1 to cells pretreated with trypsin

To ascertain whether binding of VLPs to cells required a membrane protein, several cell types were pretreated with trypsin, and the effect on VLP binding was determined. Treatment of cells with trypsin under conditions in which cell viability was unaffected generally abolished specific VLP binding: for CV-1 cells, binding of VLPs was reduced by 88% (Fig. 2). Four of sixteen receptor positive cell lines demonstrated VLP binding that was not trypsin sensitive (Table 1). Binding of Hexahis L1 to CV-1 (Fig. 2) and BL72 cells was demonstrated not to be trypsin sensitive, confirming that the Hexahis L1 and the VLP receptor are distinct on these cells.

Independence of VLP binding of membrane protein glycosylation

Tunicamycin, an inhibitor of N-glycosylation, supported growth of CV-1 cells at concentrations of less than 0.25 μg/ml. At a concentration of tunicamycin of 0.5 μg/ml or higher, growth rates of CV-1 cells were reduced. The binding of the biotin-labeled lectin Concanavalin A to CV-1 cells pretreated by tunicamycin was reduced at 0.05 μg/ml and at 0.25 μg/ml of tunicamycin mean fluorescence intensity was reduced to 40% of control values. However, binding of VLP or Hexahis L1 to CV-1 cells was not affected by pretreatment with up to 0.25 μg/ml of tunicamycin (Fig. 7).

To analyze the role of membrane protein-associated carbohydrate in binding of VLPs to cells, CV-1 cells were pretreated with neuraminidase, to remove sialic acid, and/or *O*-glycosidase. O-linked oligosaccharides were cleaved by treatment with neuraminidase and *O*-glycosidase because *O*-glycosidase acts only on desialylated oligosaccharides (Maisner *et al.,* 1994). Binding of VLP to cells was not measurably affected by pretreatment with neuraminidase or *O*-glycosidase separately. CV-1 cells treated sequentially with neuraminidase and *O*-glycosidase bound similar amounts of VLPs to control cells, but 76% of treated cells as opposed to 50% of control cells bound VLPs, suggesting that removal of membrane glycoproteins enhanced receptor availability.

DISCUSSION

In this study we have confirmed, using HPV6b L1 VLPs, the recent finding that BPV1 (Roden *et al.,* 1994) and HPV11, 16, and 33 VLPs (Muller *et al.,* 1995; Volpers *et al.,* 1995) bind to a widely distributed trypsin-sensitive cell membrane structure. We have demonstrated that VLP receptor negative cells can be found among cells from the hemopoietic lineage: previous studies have failed to identify VLP receptor negative lines and indeed cells from nonmammalian origin, including insect coelomic cells have been shown by us and by others to be

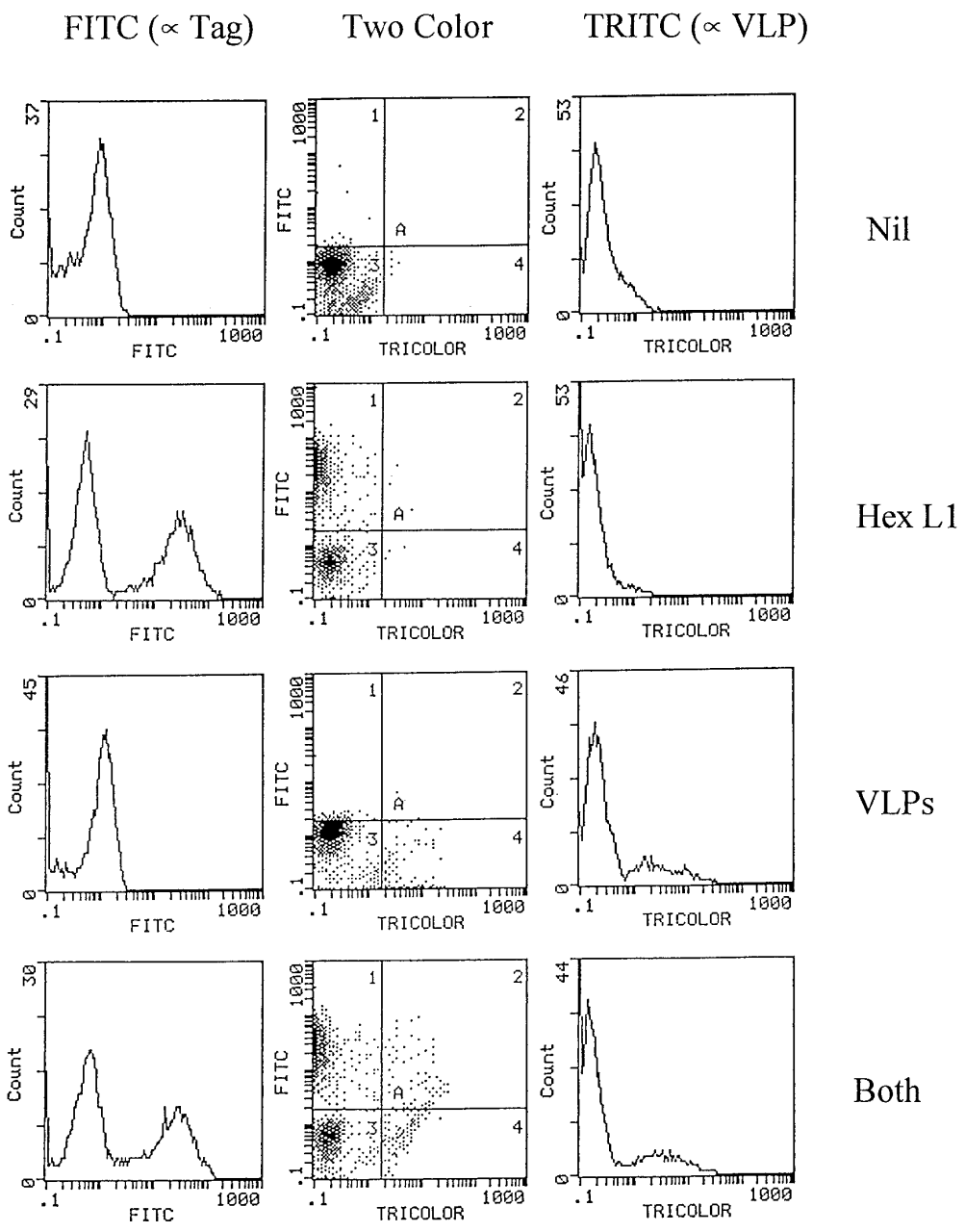

FIG. 6. Binding of HPV6b L1 VLPs and of Hexahis L1 protein to the BL72 cell line. Aliquots of BL72 cells (2×10^5) were incubated with antibodies only (Nil); 25 μg of purified Hexahis L1 protein and Hexahis-specific anti-Tag MAb (Hex L1); 25 μg of purified HPV6bL1 VLPs and rabbit anti-6bL1 antibody (VLPs); or 25 μg of both VLPs and Hexahis L1, together with rabbit anti-6L1 antibody and Hexahis-specific anti-Tag MAb (Both). All cells were then exposed to anti-mouse IgG–FITC and anti-rabbit IgG–TRITC. Flow cytometric analysis was performed for FITC alone (FITC), FITC and TRITC (Two Color), and TRITC alone (TRITC).

receptor positive. Establishment of VLP receptor negative cell lines will be critical to studies of VLP uptake, and for definition of the VLP receptor.

We have further established in this study three separate pieces of evidence that there is also a non-trypsin-sensitive cellular receptor for PV L1 protein, which binds Hexahis L1 protein but cannot bind L1 as it is configured in HPV6b L1 VLPs. These data are that (1) binding of Hexahis L1 as opposed to VLPs to CV-1 cells is to a trypsin-resistant membrane structure, (2) a cell line (DG-75) that is unable to bind VLPs is nevertheless able to bind Hexahis L1, and (3) receptors for Hexahis L1 and for VLPs are independently expressed on another cell line (BL-72).

Thus, it would appear that there may be multiple interactions between PV capsid proteins and the cell membrane. The demonstration of a cell surface structure, independent of the PV VLP receptor, which binds Hexahis L1 protein is in keeping with recent observations for a number of viruses, including adenovirus (Stevenson *et*

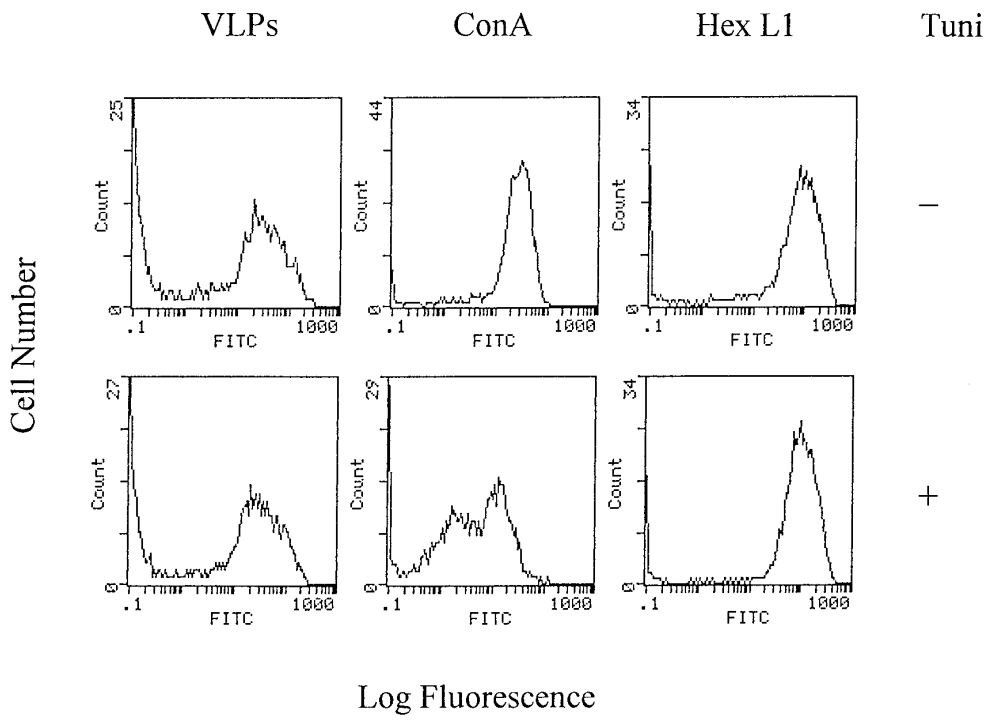

FIG. 7. Effect of tunicamycin pretreatment of CV-1 cells on the binding of HPV6bL1 VLPs or HPV6bL1 Hexahis fusion protein to cells. CV-1 cells were cultured in the absence (−) or presence (+) of 0.25 μg/ml tunicamycin for 48 hr. Cells were exposed to VLPs + Camvir 1 + anti-mouse FITC (VLPs), to biotin-labeled Concanavalin A + streptavidin FITC (Con A) or to Hexahis L1 protein + Camvir 1 + anti mouse FITC (Hex L1), and analyzed by flow cytometry.

al., 1995) and HIV-1 (Norkin, 1995), that the same virus may bind to the same or different cell types by more than one means. Presumably, such multiple interactions between the virus and cellular proteins facilitates viral entry into cells. It is unlikely that membrane-associated structures which bind L1 but not VLPs play a role in the initial binding of PV virions to cells. However, proteins of the cell membrane are recycled into the endosome along with VLP–receptor complexes (Muller *et al.,* 1995), where, by analogy with adenovirus uptake and disassembly (Greber *et al.,* 1993), an L1 binding structure might play a part in internalizing or in transport of L1–L2–DNA complexes when the PV virion is disassembled.

For our VLP and L1 receptor assays we have developed a flow cytometry-based technique, which should allow the rapid screening of sera and drugs for their ability to block binding of HPV VLPs to their target cells. It is noteworthy that although the majority of MAbs raised against an HPV16 L1 fusion protein and screened for use in this assay were able to bind HPV6b either as VLPs or as denatured L1 protein by immunoblot (data not shown), none of the antibodies were able to block binding of the VLPs, or of HPV 6bL1 protein, to the corresponding cellular receptors. This is in keeping with the observation that neutralizing antibodies are generally HPV genotype specific and HPV genotype cross-reactive antibodies are generally not neutralizing (Christensen *et al.,* 1990, 1994).

The wide distribution of the VLP receptor suggests that VLPs might bind to a posttranslational modification of a cell membrane structure, rather than a specific primary protein sequence. Papovaviridiae, including the papovavirus (Haun *et al.,* 1993; Keppler *et al.,* 1994) and most polyomavirus, e.g., human BK virus (Sinibaldi *et al.,* 1990) and mouse polyomavirus (Cahan *et al.,* 1983; Fried *et al.,* 1981; Stehle *et al.,* 1994), bind to proteins, but the state of sialation of the proteins determines receptor function (Keppler *et al.,* 1994, 1995). However, our observations on the binding of HPV6 VLPs to epithelial cells are consistent with those for the binding of HPV33 to HeLa cells (Volpers *et al.,* 1995) and the receptor of SV40 to Vero cells (Clayson and Compans, 1989), in that the state of glycosylation and sialation of the cell surface proteins does not appear to be important in determining either VLP or L1 binding.

Another posttranslational addition to cell surface proteins which could assist VLP binding to cells is fatty acylation. Fatty acids are added to cell membrane proteins either as long-chain fatty acids or as complex glycolipids (Casey, 1995). Such residues are often membrane inserted and concerned more with signaling than with ligand interaction — however, some are functional as ligand receptors. Some cells in the present study bound VLPs using a trypsin-resistant mechanism, suggesting that there might be a receptor which did not include an exposed protein component, and similarly soluble L1

binding was not trypsin sensitive. Demonstration in this present study that the majority of tested cells of the hemopoetic cell lineage were negative for receptors suggests, however, that the VLP receptor is more likely to be a specific protein or proteins than a posttranslational modification to cell surface protein as proteins with these modifications are found on proteins of all cell membranes.

Papillomavirus VLP receptors are not particularly abundant on the cell membrane. A value of 1×10^4 VLP receptors per CV-1 cell was calculated from Scatchard analysis of binding using ^{35}S-labeled VLP. This value represents the average number of VLP bound per cell since it is not known if all the cells bind VLP equivalently. This value is directly comparable with the estimate of 2×10^4 receptors/cell obtained for HeLa cells using HPV33 VLP (Volpers *et al.,* 1995), suggesting that at least on epithelial cells such receptors are equally abundant. The molar affinity constant of binding (4×10^7 *M*) is high, especially as this is expressed per L1 molecule and the virus is 360 valent, which would significantly enhance binding. The K_a calculated from VLP displacement studies was approximately 10-fold higher (data not shown), suggesting that receptor binding may be of the "fast on–slow off" type, although an alternate explanation, not excluded by current data, would be that significant internalization of labeled VLPs can occur even at 4°. The majority of tested cells of nonepithelial lineage demonstrated less intense staining by FACS analysis than CV-1 cells, suggesting, as these experiments were conduced with saturating concentrations of VLPs, that these cells have lower VLP receptor numbers. Alternatively, it remains possible that there are multiple structures able to bind VLPs on the cell surface, only one of which is expressed on epithelial cells, and that the epithelial cell-specific receptor is the determinant of PV tropism. This argument is supported by the observation that binding of VLPs to 4 of 16 "receptor positive" cell lines was not inhibited by trypsin treatment, but further characterization of trypsin-resistant cell surface molecules binding PVs will be necessary to resolve this issue.

ACKNOWLEDGMENTS

We thank Dr. Margaret Stanley and Dr. Lionel Crawford for Camvir 1 MAb. This work was funded by grants from the National Health and Medical Research Council of Australia, Grant R01-CA 57789-01 from the National Institutes of Health, the Queensland Cancer Fund, the Mayne Bequest, and the Princess Alexandra Hospital Research and Development Foundation.

REFERENCES

Borrow, P., and Oldstone, M. B. (1992). Characterization of lymphocytic choriomeningitis virus-binding protein(s): A candidate cellular receptor for the virus. *J. Virol.* 66, 7270–7281.

Bouayyad, A., and Menezes, J. (1990). Comparative study of herpes simplex virus receptor expression on human lymphoid cells. *Virology* 179, 905–910.

Cahan, L. D., Singh, R., and Paulson, J. C. (1983). Sialyoligosaccharide receptors of binding variants of polyoma virus. *Virology* 130, 281–289.

Casey, P. J. (1995). Protein lipidation in cell signaling. *Science* 268, 221–225.

Christensen, N. D., Kreider, J. W., Cladel, N. M., Patrick, S. D., and Welsh, P. A. (1990). Monoclonal antibody-mediated neutralization of infectious human papillomavirus type 11. *J. Virol.* 64, 5678–5681.

Christensen, N. D., Kirnbauer, R., Schiller, J. T., Ghim, S. J., Schlegel, R., Jenson, A. B., and Kreider, J. W. (1994). Human papillomavirus types 6 and 11 have antigenically distinct strongly immunogenic conformationally dependent neutralizing epitopes. *Virology* 205, 329–335.

Clayson, E. T., and Compans, R. W. (1989). Characterization of simian virus 40 receptor moieties on the surface of Vero c1008 cells. *J. Virol.* 63, 1095–1100.

Dalgleish, A. G., Beverley, P. C. G., Clapman, P. R., Crawford, D. H., Greaves, M. F., and Weiss, R. A. (1984). The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature (London)* 312, 763–767.

Fingeroth, J. D., Weiss, J. J., Tedder, T. F., Strominger, J. L., Biro, P. A., and Fearon, D. T. (1984). Epstein–Barr virus receptor of human B lymphocytes is the C3d receptor CR2. *Proc. Natl. Acad. Sci. USA* 81, 4510–4514.

Fried, H., Cahan, L. D., and Paulson, J. C. (1981). Polyoma virus recognizes specific sialyloligosaccharide receptors on host cells. *Virology* 109, 188–192.

Greber, U. F., Willetts, M., Webster, P., and Helenius, A. (1993). Stepwise dismantling of adenovirus 2 during entry into cells. *Cell* 75, 477–486.

Haun, G., Keppler, O. T., Bock, C. T., Herrmann, M., Zentgraf, H., and Pawlita, M. (1993). The cell surface receptor is a major determinant restricting the host range of the B-lymphotropic papovavirus. *J. Virol.* 67, 7482–7492.

Heifetz, A., Keenan, R. W., and Elbein, A. D. (1979). Mechanism of action of tunicamycin on the UDP-GlcNAc:Dolichyl-phosphate GlcNAc-1-phosphate transferase. *Biochemistry* 18, 2187–2192.

Horton, H. M., and Burand, J. P. (1993). Saturable attachment sites for polyhedrin-derived baculovirus on insect cells and evidence for entry via direct membrane fusion. *J. Virol.* 67, 1860–1868.

Keppler, O. T., Herrmann, M., Oppenlander, M., Meschede, W., and Pawlita, M. (1994). Regulation of susceptibility and cell surface receptor for the B-lymphotropic papovavirus by N glycosylation. *J. Virol.* 68, 6933–6939.

Keppler, O. T., Stehling, P., Herrmann, M., Kayser, H., Grunow, D., Reutter, W., and Pawlita, M. (1995). Biosynthetic modulation of sialic acid-dependent virus–receptor interactions of two primate polyoma viruses. *J. Biol. Chem.* 270, 1308–1314.

Kirnbauer, R., Booy, F., Cheng, N., Lowy, D. R., and Schiller, J. T. (1992). Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proc. Natl. Acad. Sci. USA* 89, 12180–12184.

Maisner, A., Schneider Schaulies, J., Liszewski, M. K., Atkinson, J. P., and Herrler, G. (1994). Binding of measles virus to membrane cofactor protein (CD46): Importance of disulfide bonds and N-glycans for the receptor function. *J. Virol.* 68, 6299–6304.

McLean, C. S., Churcher, M. J., Meinke, J., Smith, G. L., Higgins, G., Stanley, M., and Minson, A. C. (1990). Production and characterization of a monoclonal antibody to human papillomavirus type 16 using recombinant vaccinia virus. *J. Clin. Pathol.* 43, 488–492.

Muller, M., Gissmann, L., Cristiano, R. J., Sun, X. Y., Frazer, I. H., Jenson, A. B., Alonso, A., Zentgraf, H., and Zhou, J. (1995). Papillomavirus capsid binding and uptake by cells from different tissues and species. *J. Virol.* 69, 948–954.

Norkin, L. C. (1995). Virus receptors: Implications for pathogenesis and the design of antiviral agents. *Clin. Microbiol. Rev.* 8, 293–315.

Park, D. S., Selvey, L. A., Kelsall, S. R., and Frazer, I. H. (1993). Human

papillomavirus type 16 E6, E7 and L1 and type 18 E7 proteins produced by recombinant baculoviruses. *J. Virol. Methods* 45, 303–318.

Roden, R. B., Kirnbauer, R., Jenson, A. B., Lowy, D. R., and Schiller, J. T. (1994). Interaction of papillomaviruses with the cell surface. *J. Virol.* 68, 7260–7266.

Sahli, R., Freund, R., Dubensky, T., Garcea, R., Bronson, R., and Benjamin, T. (1993). Defect in entry and altered pathogenicity of a polyoma virus mutant blocked in VP2 myristylation. *Virology* 192, 142–153.

Schindler, P. A., Settineri, C. A., Collet, X., Fielding, C. J., and Burlinggame, A. L. (1995). Site-specific detection and structural characterization of the glycosylation of human plasma proteins lecithin: Cholesterol acyltransferase and apolipoprotein D using HPLC/electrospray mass spectrometry and sequential glycosidase digestion. *Protein Sci.* 4, 791–803.

Sinibaldi, L., Goldoni, P., Pietropaolo, V., Longhi, C., and Orsi, N. (1990). Involvement of gangliosides in the interaction between BK virus and Vero cells. *Arch. Virol.* 113, 291–296.

Stehle, T., Yan, Y., Benjamin, T. L., and Harrison, S. C. (1994). Structure of murine polyomavirus complexed with an oligosaccharide receptor fragment. *Nature* 369, 160–163.

Stevenson, S. C., Rollence, M., White, B., Weaver, L., and McClelland, A. (1995). Human adenovirus serotypes 3 and 5 bind to two different cellular receptors via the fiber head domain. *J. Virol.* 69, 2850–2857.

Tindle, R. W., Smith, J. A., Geysen, H. M., Selvey, L. A., and Frazer, I. H. (1990). Identification of B epitopes in human papillomavirus type 16 E7 open reading frame protein. *J. Virol.* 71, 1347–1354.

Volpers, C., Unckell, F., Schirmacher, P., Streech, R. E., and Sapp, M. (1995). Binding and internalization of human papillomavirus type 33 virus-like particles by eukaryotic cells. *J. Virol.* 69, 3258–3264.

Wickham, T. J., Mathias, P., Cheresh, D. A., and Nemerow, G. R. (1993). Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell* 73, 309–319.

Wong-Staal, F., and Gallo, R. C. (1985). Human T-lymphotropic retroviruses. *Nature (London)* 317, 395–403.

Zhou, J., Sun, X. Y., Stenzel, D. J., and Frazer, I. H. (1991). Expression of vaccinia recombinant HPV 16 L1 and L2 ORF proteins in epithelial cells is sufficient for assembly of HPV virion-like particles. *Virology* 185, 251–257.

Zhou, J., Stenzel, D. J., Sun, X.-Y., and Frazer, I. H. (1993). Synthesis and assembly of infectious bovine papillomavirus particles *in vitro*. *J. Virol.* 74, 763–768.